Int. J. Dev. Biol. 64: 331-341 (2020)
https://doi.org/10.1387/ijdb.190190mt

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

Transcriptome profiling identifies multistep regulation through *E93, Forkhead* and *Ecdysone Oxidase* in survival of Malpighian tubules during metamorphosis in *Drosophila*

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ABSTRACT *Drosophila* metamorphosis is associated with substantial metabolic activity involving cell death and cell proliferation leading to differentiation of adult tissues and structures. Unlike other larval tissues, Malpighian tubules (MTs) exhibit apoptotic immunity and do not undergo cell death but are carried over to the adult with some cell reorganisation. They persist despite the fact that they express apoptotic proteins and caspases. In the present study, we analysed the global transcription changes in MTs and compared with salivary glands, to decipher the biology of MTs. Gene set enrichment analysis indicated reduced expression of many ecdysone induced genes, including the critical regulator of cell death, E93 in MTs. We hypothesize that reduction of E93 could be because of over expression of ecdysone oxidase, which is high in MTs and is responsible for regulation of hormone titer by degradation of ecdysone. Ectopic expression of E93 in MTs results in cell death through autophagy. Fork head, which is crucial for survival, is enriched in the MT transcriptome, and its down regulation in MTs could be consequent to over expression of E93. Together our data suggests that the cascade of events initiated by ecdysone mediates survival of MTs through concerted action of multiple factors.

KEY WORDS: *Drosophila, E93, ecdysone, Malpighian tubules, programmed cell death*

Introduction

Drosophila development is incomplete without absolute metamorphosis of crawling and feeding larva to highly motile adult during pupation. Metamorphosis involves cell death of most/many of the larval tissues such as salivary glands, midgut, and fat bodies which is brought about by coordinated action of apoptosis and autophagy (Baehrecke, 2005; Jiang *et al.,* 1997; Lee *et al.,* 2000), except some tissues such as MTs which escape cell death. These dynamic changes are governed by steroid hormone 20-hydroxy ecdysone (ecdysone) which is released at regular intervals during late larval and pupal stages of *Drosophila*, and regulates a large number of primary and secondary response genes.

The signaling cascade initiates when ecdysone binds to a heterodimeric receptor complex comprising of ecdysone receptor and ultraspiracle and directly induces transcription of ecdysone responsive primary genes, *BR-C* (Broad Complex), *E74A*, *E75* and *E93* (Baehrecke and Thummel, 1995; Burtis *et al.,* 1990; DiBello *et al.,* 1991; Koelle *et al.,* 1992; Segraves and Hogness, 1990). These genes encode transcription factors that facilitate the induction of a large set of secondary late response genes which coordinate apoptosis of larval tissues and proliferation and formation of adult structures (Baehrecke, 2000; Thummel, 1996). Among the ecdysone induced primary genes, *E93* has a critical role in ecdysone induced apoptosis and autophagy in the fat body, midgut, and salivary glands (Baehrecke *et al.,* 2000; Liu *et al.,* 2015).

Apoptosis and autophagy are the two important programs that cause developmental cell death in *Drosophila* (Baehrecke, 2002; Denton *et al.,* 2012). Activation of caspase family of cysteine proteases is the decisive factor in apoptotic pathway with Dronc being the initiator caspase and Drice the major effector caspase (Steller, 2008). Dronc is activated following dimerization and recruitment

Abbreviations used in this paper: BR-C, broad complex; EcR, ecdysone receptor; Eigs, ecdysone induced secondary genes; GO, gene ontology; h AEL, hours after egg laying; h APF, hours after puparium formation; LMT, larval Malpighian tubule; LSG, larval salivary gland; MT, Malpighian tubule; PCD, programmed cell death; PMT, pupal Malpighian tubule; PSG, pupal salivary gland; SG, salivary gland.

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Supplementary Material (five tables) for this paper is available at: https://doi.org/10.1387/ijdb.190190mt

Submitted: 11 July, 2019; *Accepted:* 19 August, 2019.

ISSN: Online 1696-3547, Print 0214-6282

Printed in Spain

into an apoptosome with the help of adaptor protein, Dark/Ark, a CED4/Apaf-1 homologue which is independent of cytochrome c (Dorstyn and Kumar, 2006; Rodriguez *et al.,* 1999). In *Drosophila,* the caspases are kept in check by Inhibitor of Apoptosis family of proteins, DIAP1 and DIAP2 (Duckett *et al.,* 1996; Hay *et al.,* 1995). Apoptosis ensues when IAP-dependent inhibition of caspases is abolished by IAP-antagonists *reaper*, *hid* and *grim* residing at the *H99* Locus (Chen *et al.,* 1996; Grether *et al.,* 1995; White *et al.,* 1994). These IAP-antagonists bind to DIAP1 disrupting its interaction with caspases and initiating caspase activation (Ryoo *et al.,* 2002; Yoo *et al.,* 2002). On the other hand autophagy is dependent upon activation of genes such as *Atg-2, Atg-4, Atg-5, Atg-6, Atg-7, Atg-9* and *Atg-12* (Das *et al.,* 2012; Gorski *et al.,* 2003; Xu *et al.,* 2015). An additional pathway of inhibiting apoptosis is by activation of ecdysone regulated transcription factor, *Fork head* (*Fkh*). Reduction in Fkh is quintessential to cause death in salivary glands, and there is concomitant drop in its expression in salivary glands from late third instar larval stage, making them competent to respond to ecdysone-induced death signals (Cao *et al.* 2007). Repression of reaper and hid transcription by Fkh restrains activation of apoptosis, as well as downregulation of the entire cassette of autophagy genes (Lehmann, 2008).

The ecdysone titer exhibits distinct peaks at specific stages in development and precise pulses of ecdysone are regulated by its biosynthesis and catabolism. Ecdysone oxidase (EO) is an inactivating enzyme which catalyses the oxidation of ecdysone, inhibiting its activity (Li *et al.,* 2015; Takeuchi *et al.,* 2005). *Drosophila* MTs are equivalent to mammalian kidneys, responsible for excretion of toxic wastes and osmoregulation. Apart from these they are also immune responsive tissues and secrete Anti-Microbial Peptides (AMPs) to fight against pathogen infections (McGettingan *et al.,* 2005; Verma and Tapadia, 2012) and are developmentally regulated (Verma and Tapadia, 2015). What makes MTs stand apart is that it does not undergo cell death during metamorphosis when most of the other tissues do. This became all the more intriguing as they do express apoptotic proteins, and they show very different morphological and physiological response to ecdysone (Shukla and Tapadia, 2011; Tapadia and Gautam, 2011). Salivary glands (SGs) cells initiate the process of programmed cell death (PCD) while MTs cell continues to exist in adults. How a particular systemic signal (ecdysone pulse) executes PCD in one tissue and not in the other, offers an opportunity to study tissue specific transcriptomic activity. Tissue specific differences is highly dependent on gene function so we aimed to identify the genes which give rise to different fates of MTs and SGs. In this study we have identified genes that are differentially expressed in MTs as compared to SGs during 3rd instar larval (110 h AEL) and 12 h APF pupal stage when ecdysone triggers metamorphosis. On the basis of differentially expressed genes we have hypothesised a pathway where we categorically show that E93, Fkh and EO play a critical role in altering the fate of MTs.

Results

Active caspases in Malpighian tubules do not induce apoptosis

As MTs do not undergo cell death during metamorphosis despite the presence of caspases (Shukla and Tapadia, 2011), we wanted to check caspase activity, for which we used *in-vivo* dual color Caspase Tracker biosensor (Tang *et al.,* 2016). By this technique, cells expressing transient red fluorescent protein (RFP) is indicative of recent or on-going caspase activity whereas past caspase activity, not amounting to death, can be identified by green fluorescent protein (GFP). It was noteworthy that in MTs of 2 day old adult flies red fluorescence (Fig. 1A) as well as green fluorescence (Fig. 1B) showed that cells remained alive even in presence of active caspases. The evidence that active caspase was present earlier and is still persisting was confirmed by cells that were emitting yellow fluorescence (Fig. 1D).

Having observed active caspase in MTs, we further wanted to check if it does function to activate apoptosis in MTs or not? Cells in process of apoptosis or type I PCD exhibit well defined characteristic such as membrane blebbing, cell shrinkage, chromatin breakdown and disruption of nuclear membrane integrity (Kerr *et al.,* 1972). As degradation of Lamins by Caspase-3 causes nuclear blebbing (Kivinen *et al.,* 2005; Kihlmark *et al.,* 2001), we first confirmed the integrity of nuclear membrane in MTs and compared with SGs on account of them undergoing apoptosis. Immunostaining with Anti Lamin-B antibody in 3rd instar larval (110-116 h AEL) tissues revealed sharp, intact and continuous nuclear membrane in the larval MTs (Fig. 2A) as well as in the larval SGs (Fig. 2B) confirming the absence of apoptosis in both the tissues. However, pupal SGs (12-14 h APF) acquire morphological abnormalities as the nuclear membrane appeared discontinuous (Fig. 2D) indicating that they are undergoing PCD, whereas nuclear membrane in MTs appeared to be continuous and intact (Fig. 2C), and same as observed at the larval stage. These results confirmed that the MTs do not un-

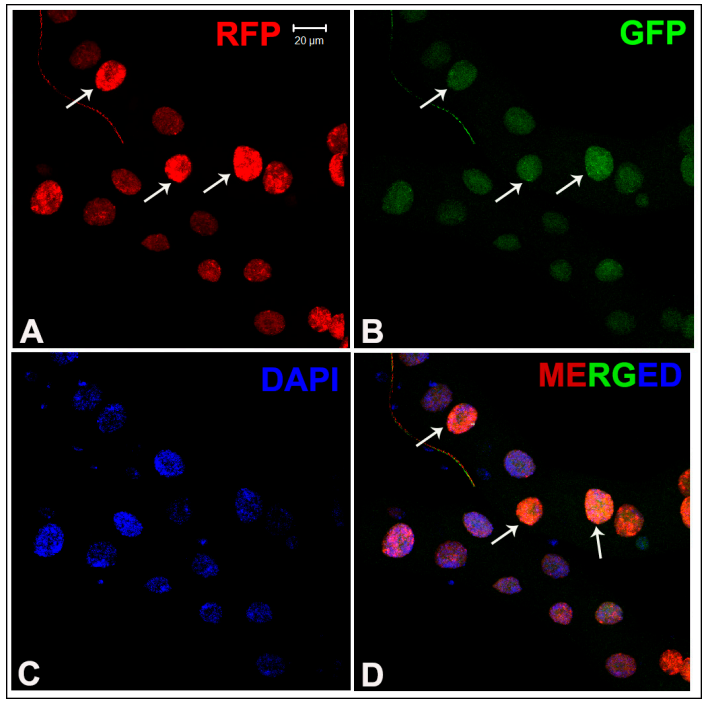

Fig. 1. Caspase activity in Malpighian tubules (MTs). *Dual colour Caspase Tracker biosensor showing expression of transient caspase activity with red fluorescence* **(A)** *RFP, past caspase activity is shown with green fluorescence* **(B)** *GFP and chromatin was stained with DAPI* **(C)** *in 2 day old adult MTs. The merged panel* **(D)** *shows the overlap of past and present caspase activity in the nucleus. Arrows indicate the cells showing both past and transient caspase activity. All images are projection of optical sections obtained by Zeiss LSM 510 Meta Confocal microscope. Scale bar, 20 µm.*

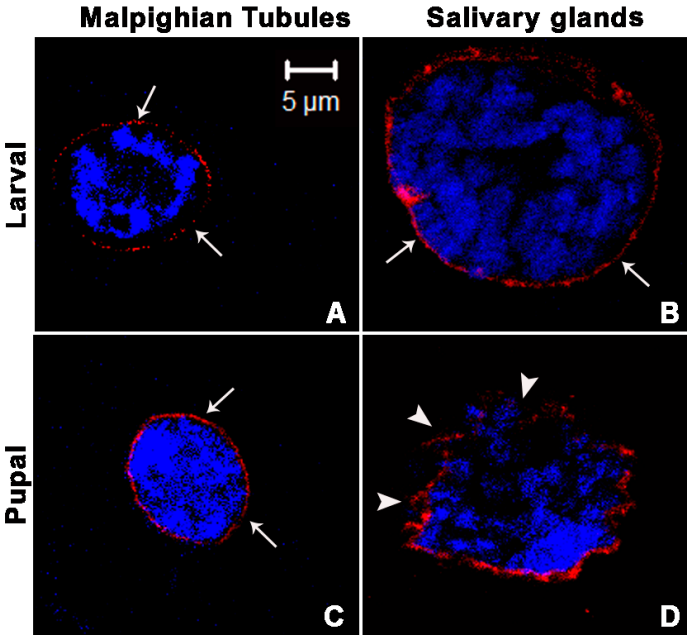

Fig. 2. Absence of nuclear membrane blebbing in Malpighian tubules (MTs) at pupal stage showing absence of cell death. *MTs and salivary glands (SGs) immunostained with antibody against Lamin-B showing intact nuclear membrane at* **(A)** *Larval (110-116 h AEL) MTs;* **(B)** *larval (110-116 h AEL) SGs and* **(C)** *12-14 h APF pupal MTs (arrows). The disrupted nuclear membrane was observed in* **(D)** *12-14 h APF pupal SGs (arrow head). Chromatin was stained with DAPI which clearly shows DNA within the nuclear periphery. All images are single optical section obtained by Zeiss LSM 510 Meta Confocal microscope. Scale bar, 5 µm.*

dergo ecdysone induced apoptosis which is consistent with the earlier report that MTs escapes apoptosis during metamorphosis (Shukla and Tapadia, 2011). These findings provide unequivocal evidence that MTs do not undergo apoptosis in spite of the presence of active caspases.

Temporal and spatial gene expression variability in Malpighian tubules and salivary glands

Having confirmed that the MTs do not undergo apoptosis whereas SG does, the inter-tissue variability regarding differentially expressing genes (DEG) was investigated by analysing the entire transcriptome of MTs and SGs. A number of transcriptomic studies based on spatio-temporal expression in different *Drosophila* tissues have been reported, allowing exhaustive exploration of the transcriptome across a wide range of tissues and cell types (Gorski *et al.,* 2003; Lee *et al.,* 2003; Li and white, 2003), however to our knowledge, no database exists which compares apoptotic versus non-apoptotic tissues. Apoptosis has been studied extensively in SGs, so it was used as prototype to identify DEGs and biological processes that could be involved in survival of MTs (Cao *et al.,* 2007; Jiang *et al.,* 1997; Lee *et al.,* 2000; Lee and Baehrecke, 2001). The transcriptome data for DEGs was compared at two developmental stages through pairwise comparisons. First comparison was done between larval Malpighian tubules (LMT) with pupal Malpighian tubules (PMT), and larval salivary glands (LSG) with pupal salivary glands (PSG), to identify how expression profile of each tissue changes at different developmental stages, and to obtain information about the ongoing processes required for the functioning of a particular tissue at that particular stage. The second comparison was done between MTs and SGs, at similar developmental stages i.e. at larval and pupal (LMT vs LSG and PMT vs PSG) so as to identify how the expression varies in different tissues at the same developmental stage enabling unique tissue specific responses to developmental clues.

Transcriptome was analyzed taking 4>fold difference as significant, and a comprehensive view of transcripts that show substantial diversity spatially and temporally were identified. The variation in the number of transcripts that either increased or decreased significantly between different stages is more pronounced in SGs (1115 genes = 589 upregulated + 526 downregulated) as compared to MTs (750 genes = 393 upregulated + 357 downregulated) (Fig. 3A), indicating that the SGs are undergoing drastic remodeling whereas MTs are not. Of the 1865 genes that showed significant variation in both the tissues, only 247 (15.3%) were common suggesting less functional similarity between the two tissues (Fig. 3C). Conversely the comparison between the pupal and larval stage tissues resulted in almost similar number of genes in SGs (1585) and MTs (1533) (Fig. 3 B,D) with 755 (32%) genes commonly enriched between larval and pupal stages. The higher percentage of common gene data highlights the fact that genes responsible for maintenance of specific stages were irrespective of tissue type.

Transcript enrichment for particular functional class of genes in pupal tissues in comparison to their larval counterparts was determined by Gene ontology (GO) annotation which classifies genes into groups with similar function with the help of David Bioinformatics Resources 6.8 annotation tool. Significantly upregulated and downregulated genes from pair wise comparisons of PMT vs LMT (Supplementary Table S1) and PSG vs LSG (Supplementary Table S2) were selected, which were further sorted in different pathways via GO categories, and Supplementary Table S3 shows a subset of the pathways affected. Since we wanted to identify factors respon-

TABLE 1

EXPRESSION OF ECDYSONE INDUCED SECONDARY GENES IN MALPIGHIAN TUBULES AND SALIVARY GLANDS AT 3RD INSTAR LARVAL AND 12 h APF PUPAL STAGE

Genes	PMT vs LMT	PSG vs LSG	LMT vs LSG	PMT vs PSG
Eip63E	1.82	9.79	1.91	2.95
Eip93F	16.53	53.78	2.9	-1.12
Eip75B	5.61	1.15	1.01	1.62
Eip78C	-1.94	-7.24	-9.3	-2.49
Eip55E	-1.34	-7.29	-1.21	4.45
Eip63F-1	7.23	-47.7	-6.9	50.06
Ftz-f1	38.17	60.63	1.02	1.31
BR-C	1.70	1.20	1.02	1
Fkh	1.89	-4.69	6.16	54.66
EO	2.32	-1.58	25.09	91.77
Eig71Ej	1.02	2873	-1.59	-1141
Eig71Ek	-1.12	2783	-1.38	-4326
Eig71Ei	-1.07	1161	-1.48	-1831
Eig71Eh	1.08	479	-1.68	-744
Eig71Ef	-1.1	33	-164	-5989
Eig71Ed	-1.18	17.62	-264	-5497
Eig71Ec	-1.04	14.27	-314	-4669
Eig71Eg	1.03	7.95	-553	-4287
Eig71Ea	-1.03	7.92	-526	-4310
Eig71Eb	-1.12	7.08	-571	-4535
Eig71Ee	-3.92	-18.99	-654	-134

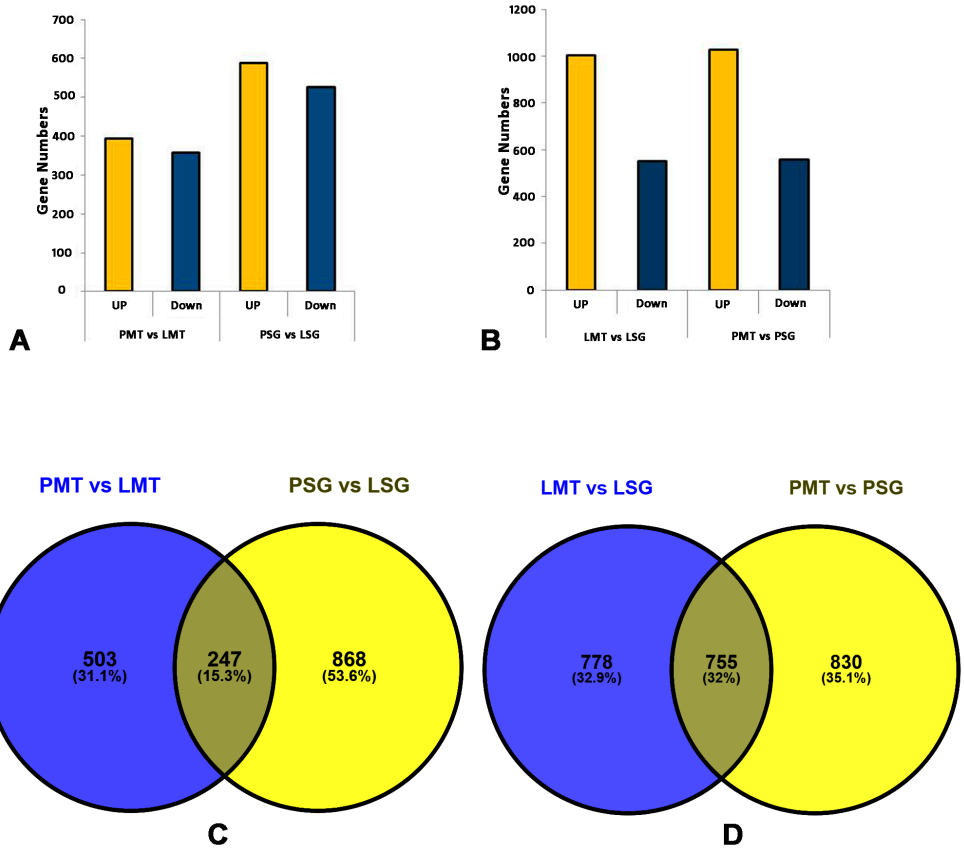

Fig. 3. Differential gene expression in Malpighian tubules (MTs) and salivary glands (SGs) at different stages. *Graph showing number of genes with significantly upregulated and downregulated transcripts in* **(A)** *PMT vs LMT and PSG vs LSG,* **(B)** *PMT vs PSG and LMT vs LSG. The number of commonly altered genes between the two comparisons is shown by Venn diagram. The common gene number is low in PMT vs LMT (Blue) and PSG vs LSG (Yellow)* **(C)** *compared to LMT vs LSG (Blue) and PMT vs PSG (Yellow)* **(D)**.

sible for absence of cell death in MTs, we chose transcripts which belonged to specific GO categories such as Autophagy cell death, Apoptosis, Salivary gland cell death, Metamorphosis, Instar larval or pupal development (Supplementary Table S4) and a heat map illustrating differential expression patterns from the two tissues at larval and pupal stages was constructed (Fig. 4; Supplementary Table S5). The widespread differences in gene expression among the two tissues were distinctly evident.

Evaluation of ecdysone regulated programmed cell death genes in Malpighian tubules and salivary glands

The hierarchy of early and late gene expression correlates with the ecdysone titer during metamorphosis (Jiang *et al.,* 2000; Thummel, 1996). Comprehensive view of ecdysone regulated DEGs revealed profound differences between the two tissues under consideration (Table 1). A significant down regulation of most secondary genes in PMTs under all comparisons was observed and a significant number of genes showed more than 1000 fold down regulation when compared to PSGs. Given the large number of ecdysone induced secondary genes (Eigs) repressed in PMTs in comparison to PSGs gave the first indication that ecdysone signaling is negatively regulated in MTs at pupal stage but it is not completely abolished. The down regulation could not be due to unavailability of Ecdysone receptor (EcR), as robust expression of EcR is observed in MTs (Gautam *et al.,* 2015) and hence the possible reason for disruption of ecdysone signaling could be reduced ecdysone availability in MTs. This inference was supported by the observation that there was significant upregulation of *EO* in MT in comparison to SG (Table 1). EO which is responsible for regulating the ecdysone levels (Li *et al.,* 2015) is possibly reducing the availability of ecdysone in MTs.

We then checked the expression of few selected genes *viz E93, BR-C* and *Fkh*, that are expressed in response to late larval and pre-pupal ecdysone pulse and their expression directly triggers the cell death cascade (Jiang *et al.,* 1997; Cao *et al.,* 2007; Lee *et al.,* 2000; Lehmann, 2008) (Table 1). Among the early genes, notable was the expression of E93, which is considered as critical regulator of PCD, and was significantly upregulated in SGs as reported earlier (Baehrecke and Thummel, 1995; Lee *et al.,* 2000), and its expression was substantially reduced in MTs implicating that this is possibly one of the determining factors responsible for the survival of MTs. Although the function of BR-C has been implicated in apoptosis (Emery *et al.,* 1994; Restifo and White, 1992) and activation of Dronc (Cakouros *et al.,* 2002), no difference was observed in the expression in SGs and MTs, suggesting that BR-C alone is not

TABLE 2

VALIDATION OF SELECTED GENES FROM MICROARRAY DATA BY QRT-PCR. GENE EXPRESSION IS SHOWN AS MEAN FOLD (+SD) CHANGE

Genes	PMT vs LMT	PSG vs LSG	LMT vs LSG	PMT vs PSG
E93	59.7±4.1633	1024±28.7286	1.5±0.2676	-11.3±1.054
BR-C	1.23±0.0673	-2.14±0.0521	1.86±0.37533	1.07±0.0667
Fkh	1.14±0.0932	-3.24±0.4332	2.14±0.7423	6.06±1.1523
EO	1.14±0.0742	1.07±0.0946	13.9±2.031	14.9±1.6632

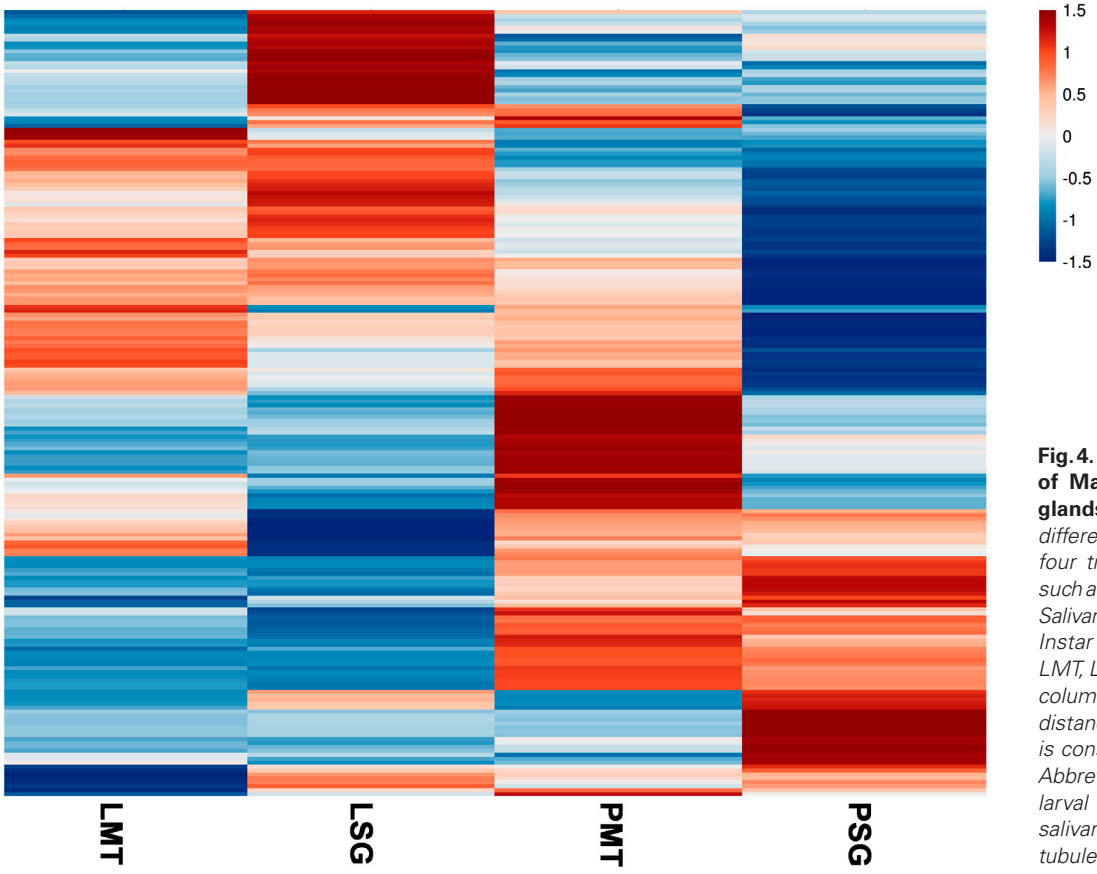

Fig. 4. Stage specific expression profile of Malpighian tubules and Salivary glands. *The heat map shows distinct differences in the transcriptome of the four tissues in specific GO categories such as Autophagy cell death, Apoptosis, Salivary gland histolysis, Metamorphosis, Instar larval or pupal development in LMT, LSG, PMT and PSG. Both rows and columns are clustered using correlation distance and average linkage. Heat map is constructed using ClustVis web tool. Abbreviations: GO, gene ontology; LMT, larval Malpighian tubules; LSG, larval salivary gland; PMT, pupal Malpighian tubule; PSG, pupal salivary gland.*

sufficient to trigger the apoptotic cascade but in conjunction with other factors including E93, it probably triggers larval cell death (Jiang *et al.,* 1997). Considering the role of BR-C in PCD regulation this result was quite surprising, suggesting strongly that ecdysone induced gene regulation is highly tissue specific and a common paradigm of PCD cannot be drawn.

A third important observation was the expression of *Fkh*, known for its role in cell survival (Cao *et al.,* 2007; Thummel, 2007) and as expected, the expression of *Fkh* was significantly downregulated in PSG in comparison to LSG and distinctly upregulated by almost 6 fold in PMTs in comparison to PSGs. In order to gain an independent assessment of expression of these genes, qRT-PCR quantification in tissue samples was performed (Table 2). These results were in complete agreement with the quantitative estimation derived from microarray analysis. Both analyses indicated that expression of *Fkh* and *EO* was significantly high in MTs in comparison to SGs and significantly low expression of E93 in PMTs in comparison to PSGs.

Having observed the induction kinetics of *EO*, *E93* and *Fkh*, and found them in accordance with earlier reports, we then examined the expression of pro-apoptotic genes viz., *reaper, grim, drice, dronc* and *dark* in MTs and SGs at larval and pupal stages. Comparative expression of the apoptotic genes in MTs and SGs was not entirely unexpected as it has been shown earlier that apoptotic proteins are associated in non-apoptotic manner in the development and function of MTs (Tapadia and Gautam, 2011). As ecdysone regulated changes in endogenous level of apoptotic genes is sufficient to explain the switch in sensitivity to apoptosis (Kang and Bashirullah, 2014), their expression in MTs raises the possibility that individual genes or gene sets are not sufficient to

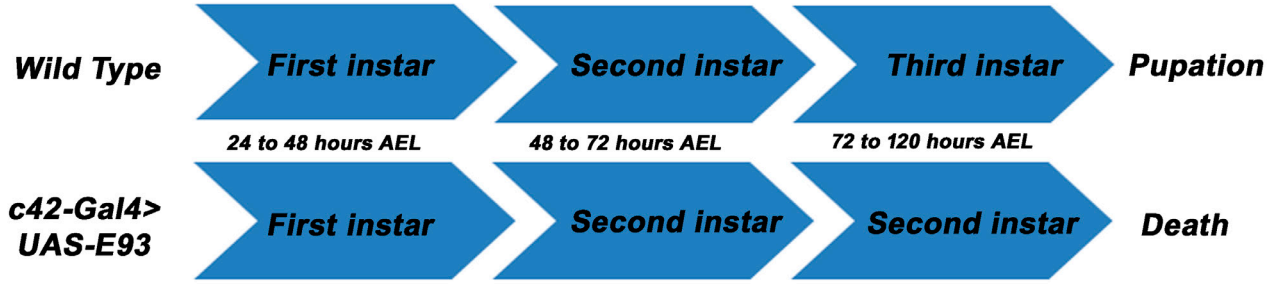

Fig. 5. Flow diagram showing difference in developmental stages between wild type and *c42-Gal4>UAS-E93* at any time point. *When wild type larvae reached third instar finally leading to pupation, the E93 over expression progenies remained in second instar resulting in death.*

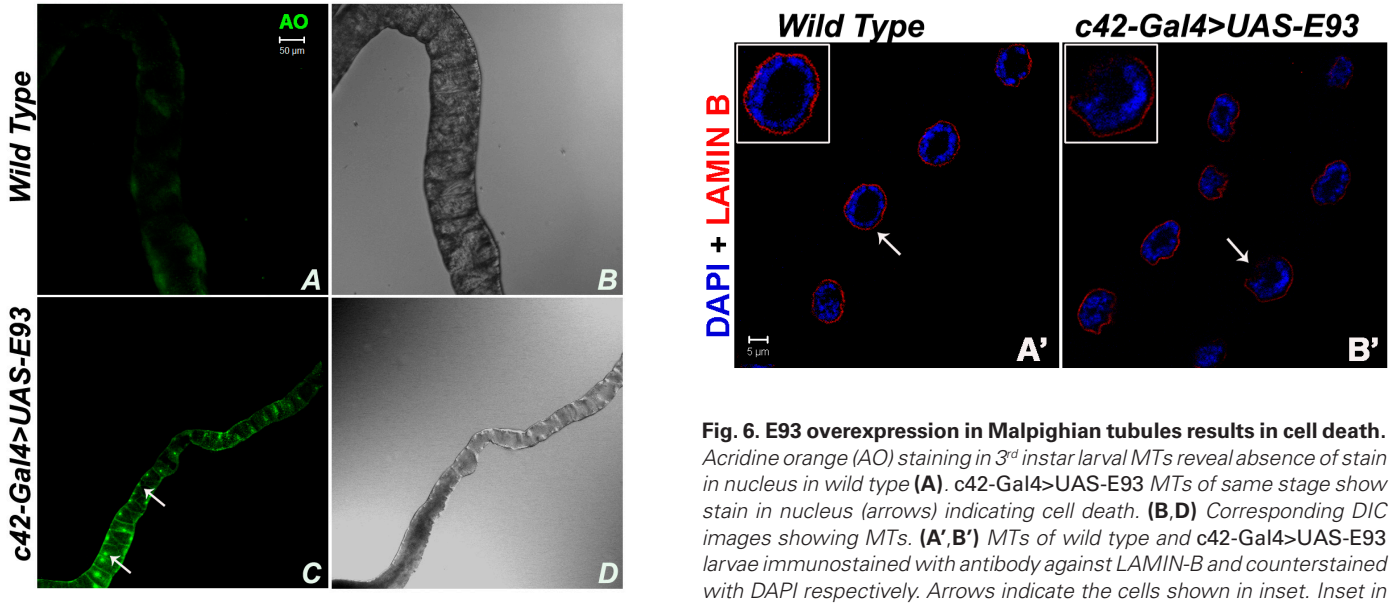

Fig. 6. E93 overexpression in Malpighian tubules results in cell death. *Acridine orange (AO) staining in 3rd instar larval MTs reveal absence of stain in nucleus in wild type* **(A)**. c42-Gal4>UAS-E93 *MTs of same stage show stain in nucleus (arrows) indicating cell death.* **(B**,**D)** *Corresponding DIC images showing MTs.* **(A'**,**B')** *MTs of wild type and* c42-Gal4>UAS-E93 *larvae immunostained with antibody against LAMIN-B and counterstained with DAPI respectively. Arrows indicate the cells shown in inset. Inset in (A' , B') shows a single nucleus at higher magnification. Images.* **(A-D)** *are projection of optical sections and images* **(A'**,**B')** *are single section obtained by Zeiss LSM 510 Meta Confocal microscope. Scale bar for images* **(A-D)** *is 50 µm and for images* **(A'**,**B')** *is 5 µm.*

understand the process that ultimately changes the fate of a tissue and that expression of apoptotic proteins is not the deciding factor to initiate PCD. One of the apoptotic gene *corp*, showed drastic upregulation in PSG compared to LSG and down regulation in PMT versus PSG (Table 3), whose significance is yet to be elucidated.

Malpighian tubule are vulnerable to cell death upon over-expression of E93

As *E93* expression is adequate to induce PCD, and to validate our hypothesis that reduced levels of *E93* is critical in determining survival of MTs, we investigated the consequences of overexpressing E93 (*UAS-E93*) using *c42-Gal4*, a MT specific Gal4 driver. *c42-Gal4>UAS-E93* larvae did not develop beyond 2nd instar larval stage and remained at the same stage for another 70-75 hours before dying. On the other hand wild type larvae developed normally and reached to 3rd instar stage (Fig. 5). As E93 over expression causes cell death (Lee *et al.,* 2000; Lee and Baehrecke, 2001; Liu *et al.,* 2015), we next assessed cell death by Acridine orange (AO) staining. AO positive cells in MTs of *c42-Gal4>UAS-E93* larvae confirmed onset of apoptosis (Fig. 6C) following over expression of E93 where as in wild type no cell death was observed (Fig. 6A). Occurrence of apoptosis was further confirmed by smooth and uniform appearance of nuclear membrane in wild type (Fig. 6A') compared to an irregular and interrupted nuclear membrane in E93 overexpression cells (Fig. 6B'). These results together validate our hypothesis that maintaining reduced expression of E93 in MT is critical for their survival.

Cell death on ectopic expression of E93 occurs through autophagy and not apoptosis

To elucidate whether apoptosis or autophagy is causing cell death in MTs of *c42-Gal4>UAS-E93* larvae, the transcript levels of key apoptotic and autophagic genes were analysed by RT-PCR. Because of developmental delay in *c42-Gal4>UAS-E93* the expression of selected genes were measured at two stages, one at 48-50 h AEL (early E93) which is equivalent to 2nd instar of wild type (early WT) and the other at 110-112 h AEL (late E93) which is equivalent to 3rd instar of wild type (late WT). Similar to expression observed in Table 3, we observed equivalent expression of pro-apoptotic genes, *reaper, hid, dark, dronc* and *drice* in early E93 and early WT as well as late E93 and late WT (Table 4) confirming

TABLE 3

EXPRESSION OF APOPTOSIS-RELATED GENES IN MALPIGHIAN TUBULES AND SALIVARY GLANDS AT 3RD INSTAR LARVAL AND 12 h APF PUPAL STAGE

Genes	PMT vs LMT	PSG vs LSG	LMT vs LSG	PMT vs PSG
grim	1.03	-1.02	-1.01	1.04
reaper	2.67	-2.34	-8.9	-1.42
drice	-2	-1.18	2.55	1.5
dronc	4.25	2.5	-1.47	1.15
dcp-1	-1.81	2.11	1.81	-1.26
dark	1.1	2.67	1.03	-1.49
diap-1	1.11	1.78	1.6	-1
corp	1.89	335.3	1.05	-168

TABLE 4

COMPARING GENE EXPRESSION BETWEEN *C42-GAL4>UAS-E93* AND WILD TYPE AT TWO DEVELOPMENTAL STAGES BY QRT-PCR. GENE EXPRESSION IS SHOWN AS MEAN FOLD (+SD) CHANGE

Genes	Late E93 vs Late WT	Early E93 vs Early WT
E93	17.4±1.0540	103.9±12.5830
Fkh	-3.2±0.0808	-1.6±0.3055
drice	-1.14±1.1633	-1.01±0.2886
dronc	-59.7±3.5563	-3.03±0.9144
dark	-84.4±25.3834	-3.03±1.4922
reaper	-5.2±0.7352	-2±0.04618
hid	-274±21.3078	-1.3±1.6196
EO	-2.8±0.0691	-1.4±0.5463
BR-C	1.6±0.3868	-1.41±1.2405
Atg-8	4.6±0.6942	1.7±0.8776

TABLE 5

COMPARING GENE EXPRESSION AMONG TWO DEVELOPMENTAL STAGES OF WILD TYPE AND *C42-GAL4>UAS-E93* BY QRT-PCR. GENE EXPRESSION IS SHOWN AS MEAN FOLD (+SD) CHANGE

Genes	Late WT vs Early WT	Late E93 vs Early E93
E93	4.2±0.3042	1.4±0.2372
Fkh	2.2±0.0590	-1.7±0.0753
drice	64±6.5240	-1.3±0.3605
dronc	21.11±1.0540	1.51±0.3868
dark	34.29±8.5220	1.23±0.2193
reaper	2.8±0.0809	1.07±1.5966
hid	238±71.4063	1.38±0.0953
EO	7.4±1.2503	3.6±0.6082
BR-C	-1.14±0.0950	2±0.3033
Atg-8	-2.6±0.3868	1.7±0.4666

that expression of apoptotic genes is not the only requirement for apoptosis. Interestingly we found that the expression of autophagy marker gene, *Atg-8,* was elevated at late E93 (Table 4), indicating occurrence of autopahgy in these cells in response to E93 overexpression. Further the expression of caspase-3 was found to be comparable by immunostaining in c42Gal4>UAS-E93 (Fig. 7B) and WT MTs (Fig. 7A) at 3rd instar, which verified the RT-PCR results. On comparison between the early and late development stages, expression of pro-apoptotic genes increased from 2nd instar to 3rd instar stage in wild type as well as E93 overexpressed condition (Table 5), suggesting that these genes are probably involved in an alternate function in MTs, confirming our earlier observation (Tapadia and Gautam, 2011).

Based on the gene expression data presented above we have proposed the mechanism which could be operating in two contrasting tissues, MTs and SGs (Fig. 8). We identified a set of three targets genes, *E93, EO* and *Fkh*, that could be necessary for implementing apoptosis or survival triggered by ecdysone stimulus. First check is at ecdysone titer regulation, where overexpressing EO deactivates and decreases the availability of active ecdysone in MTs. This leads to overall downregulation of ecdysone signaling cascade, specifically decreasing the expression of E93 and on the other hand increasing the expression of Fkh. Lower expression of *E93* and consistently higher expression of *Fkh* leads to inactivation of cell death genes and helps MTs to evade PCD during metamorphosis.

Discussion

The activation of apoptotic genes and caspases were thought to initiate an irreversible path of cell death, however this statement has been revisited as number of reports have shown non apoptotic functions of caspases in a variety of organisms, cell types and cellular processes, such as signaling, proliferation, differentiation, remodeling and neuronal plasticity (Aram *et al.,* 2017; Connolly *et al.,* 2014; Nakajima and Kuranaga, 2017). *Drosophila* MTs are the recent addition to this list as they show a robust expression of caspases but do not undergo cell death. Though tissue specificity depends on the gene expression, we recognise that just gene expression by itself is not an adequate measure to distinguish between tissues. The transcriptome analysis has provided an unprecedented opportunity to unravel complexities of regulatory network that are important for MTs survival.

The decision to prevent MTs from undergoing apoptosis is initiated by the upregulation of *EO* which is an ecdysone-responsive gene, whose promoter contains several putative binding motifs for the products of the ecdysone-responsive 'early genes', Broad-Complex and FTZ-F1 (Takeuchi H *et al.,* 2005). Ectopic expression of EO impairs tissue degeneration in *Bombyx mori* (Li Z *et al.,* 2015) suggesting that level of EO consequentially affects ecdysone induced apoptosis. In the present context the importance of EO is evident as its expression in SGs is almost undetectable whereas in MTs it is significantly high. Consequence of reduced EO essentially

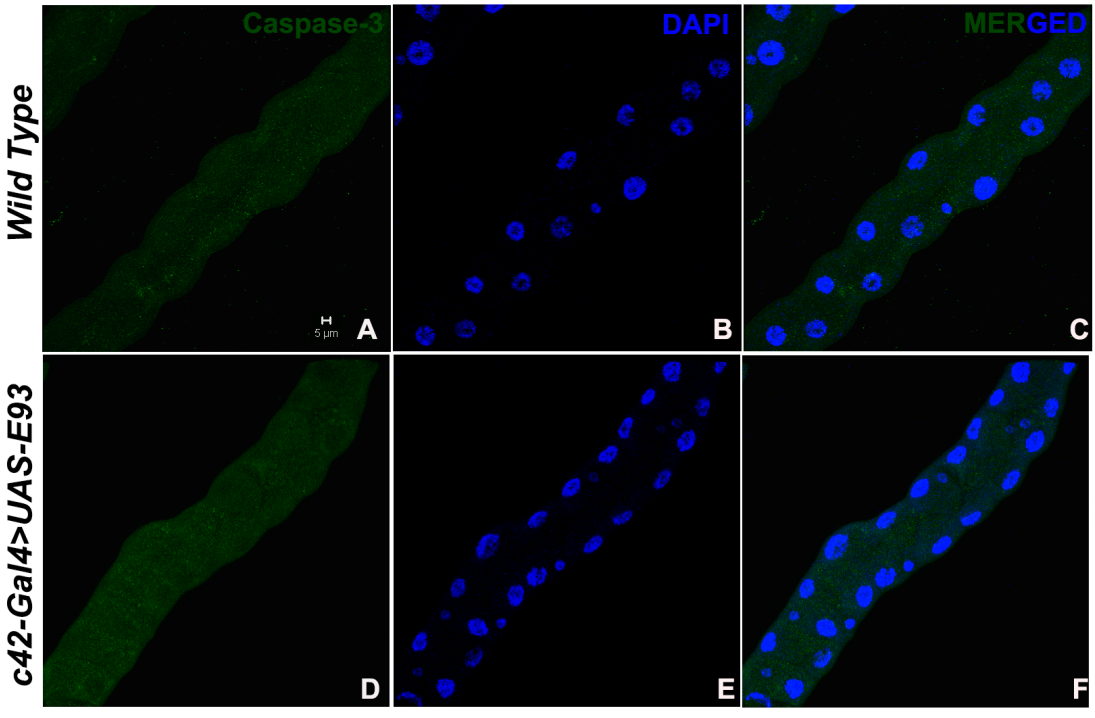

Fig. 7. Caspase-3 expression remains unchanged in *c42-Gal4>UAS-E93* Malpighian tubule with respect to wild type. *MTs of* **(A)** *wild type and* **(D)** c42-Gal4>UAS-E93 *larvae immunostained with antibody against caspase-3 and counterstained with DAPI* **(B,E)**. **(C,F)** *are merge images. All images are projection of optical sections obtained by Zeiss LSM 510 Meta Confocal microscope. Scale bar, 5µm.*

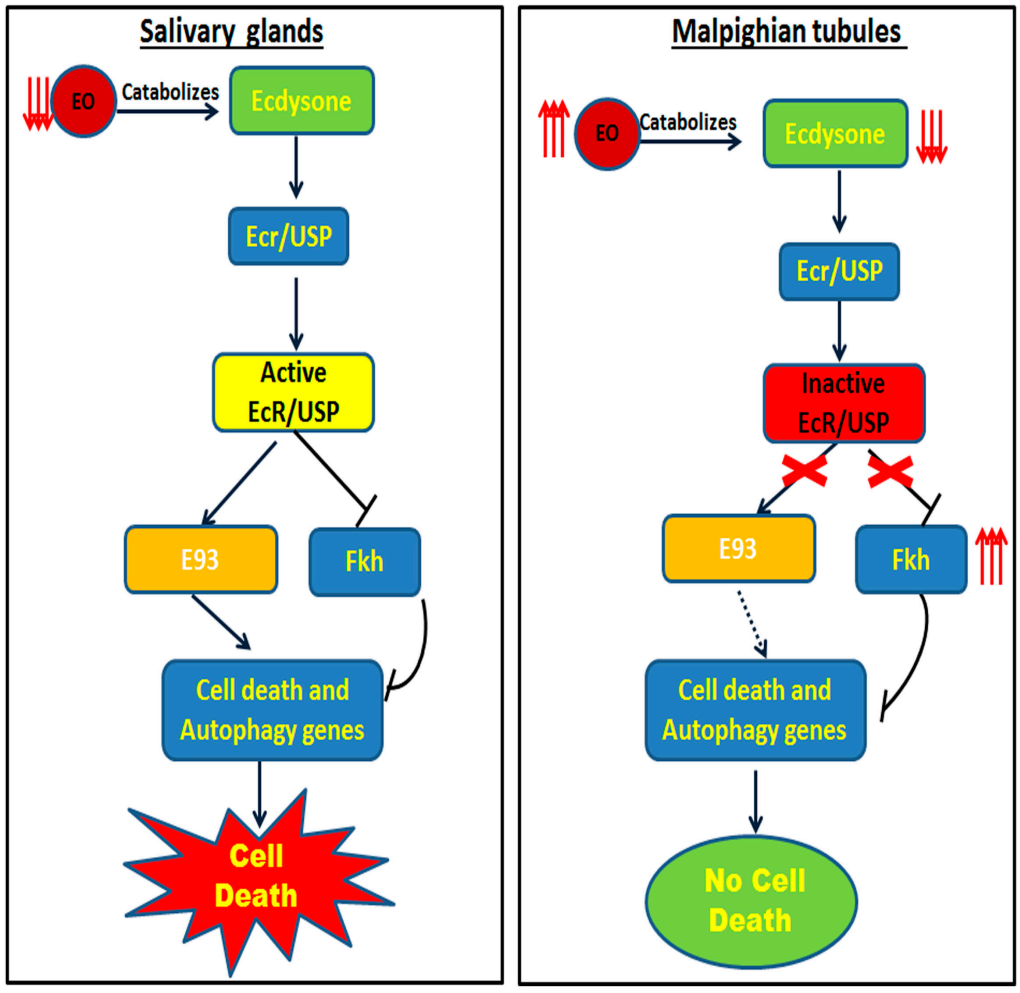

Fig. 8. Schematic representation based on microarray data, depicting factors that help Malpighian tubules to evade cell death during metamorphosis.

increases ecdysone regulated genes in SGs by almost 1000 fold for most of the secondary genes (Table 1). Among the ecdysone regulated genes noteworthy is the expression of E93, a regulator of ecdysone induced cell death (Lee *et al.,* 2000; Liu *et al.,* 2015), whose expression is very much reduced in MTs compared to SGs. Unexpectedly however, expression of E93 is not totally abolished in MTs. Though recognized for its role in cell death, E93 makes *Dll* competent to respond to EGFR signaling in bract cells during development and it also alters global responsiveness of many genes for pattern formation during metamorphosis (Mou *et al.,* 2012) suggesting its diverse function. E93 mutants are also not able to open chromatin for accessibility to transcription factors confirming its role in developmental timing through regulation of chromatin accessibility as observed in *Drosophila* wing (Praggatis and Thummel, 2017; Uyehara *et al.,* 2017). The expression of E93 in MTs suggests that it could be playing some role in the immune response (Unpublished data, Ojha and Tapadia), which is an additional function of MTs, apart from osmoregulation (McGettingan *et al.,* 2005; Verma and Tapadia, 2012). The fact that MTs continues to express AMPs even at the pupal stage (Verma and Tapadia 2012) unlike other organs, where AMP production ceases under high ecdysone titre during metamorphosis, suggests that E93 is probably endowed with two tasks, one preventing apoptosis and second regulating immunity. Reduced expression of E93 could be a tradeoff that has evolved considering its requirement for the expression of antimicrobial peptides (Unpublished data, Ojha and Tapadia). These results suggest that context-dependent regulatory paths delineate tissue specificity and this strategy reinforces a link between survival and immune response in MTs. In this paper, we have partly unraveled the reason for long standing question as to why MTs are not histolysed, while other larval tissues are. Interestingly BR-C, which is important for steroid induced cell death in dying larval tissues (Restifo and White, 1992) is not down-regulated in MTs, but shows similar expression as observed in SGs. This contradiction is acceptable, because in MTs the immune cascade is also under the regulation of BR-C (Verma and Tapadia, 2015) and therefore MTs cannot afford to downregulate BR-C. These results show that sensitivity of the tissue to a particular fate is highly dynamic and highly dependent on spatial and temporal regulators. Combinatorial activation and deactivation of different regulatory factors at specific developmental stages finally modulate the end result. Simultaneous reduction of Fkh in SGs is consistent with the earlier report that knockdown of Fkh leads to premature salivary gland death in response to the late-larval 20E pulse (Cao *et al.,* 2007; Myat and Andrew, 2000) thus its presence in MTs is sufficient to explain its role in preventing MTs from undergoing cell death.

The MTs were categorized into one of the privileged tissues that are immune to PCD. This definition we use with caution as from the present results it is evident that under conditions of augmenting the level of E93, MTs exhibit symptoms of cell death (Fig. 6, 7). Existing knowledge till now suggests that in *Drosophila* the decision to trigger apoptosis depends on transcription of IAP antagonists *reaper, hid* and *grim*. But this does not appear to be true always because we do not observe increase in expression of apoptotic genes in spite of the cell death caused by increase in E93; rather it is due to increase in the autophagic gene *Atg-8*. Autophagy also mediates degradation of nuclear lamina by direct physical interaction with Lamin-B and LC3, showing involvement of autophagic machineries in nuclear disintegration (Dou *et al.,* 2015). In MTs, precise balance of apoptotic genes is necessary for its development and function (Tapadia and Gautam, 2011), which is also validated by the present transcriptomic data (Table 2), and the presence of active caspases. The mystery of MTs deepens because the presence of caspases is not enough to lead to apoptosis, similar to the observation that expression of caspases in early 3rd instar salivary glands does not result in apoptosis (Kang and Bashirullah, 2014). Rather than the presence or absence of activation of apoptotic machinery it is now becoming more important to identify other tissue and stage specific factors that modulate the sensitivity of the tissues (Kang and Bashirullah, 2014). The essential implications are that a multitude of factors make a tissue competent to enter PCD pathway, therefore keeping check on the amount of E93 appears to be the most important factor in their survival. MTs validate the hypothesis that microenvironment of a cell makes it responsive/sensitive to apoptosis trigger signal.

Although ecdysone is an important hormone in *Drosophila*, the array of genes regulated by it is diverse, highly tissue specific, and depends on the function of the tissue. Together our data suggest that cell death evasion by MTs is mediated by check points on multiple targets to regulate ecdysone titer and ecdysone signaling. The broader perspective of the entire data suggests that pathway specific genes do assume bottleneck positions due to variability in many factors which influence canonical/non-canonical regulatory interactions.

Materials and Methods

Drosophila *stocks and genetic crosses*

Wild type Oregon R^+, principal cell specific GAL4 driver,*c42* (a kind gift from Dr. J.A.T Dow, Institute of Biomedical Sciences, University of Glasgow, UK), Caspase Tracker (DQVD) a *mCD8-DIAP1-GAL4* driven by ubiquitin promoter (kind gift from Dr. Ho Lam Tang, The Johns Hopkins University School of Medicine, USA). UAS responders used: *yw,UAS-E93; +; +* (kind gift from Dr. Eric Baehrecke, Department of Molecular, Cell and Cancer Biology, University of Massachusetts Medical School, USA), G-TRACE-*UAS-RFP; UAS-FLP; Ubi>Stop>GFP-nls* (Bloomington Stock Centre). Appropriate crosses were set up to generate *UAS-E93; c42-GAL4* and caspase Tracker/G-trace and the progeny were used for experiments. All stocks and crosses used in this study were maintained on standard *Drosophila* food medium at 23±1 °C.

Microarray analysis

RNA was isolated from Oregon R^+ 110-112 h AEL larval SGs and MTs and 12-14 h APF pupal SGs and MTs using TriReagent (Sigma-Aldrich) as per manufacturer's instructions. Microarray analysis of these RNA samples was performed on Affymetrix *Drosophila* Genome 2.0 microarray chips for 3' IVT array following the Affymetrix GeneChip Expression Analysis Technical manual using the GeneChip 3' IVT Plus Reagent Kit, Affymetrix GeneChip® Fluidics station 450, GeneChip® Hybridization oven 645 and GeneChip®Scanner 3000. Summary of the expression levels for each gene in the four genotypes was obtained from the Affymetrix Transcription analysis console and genes were designated as changed significantly if their measurement were greater than 4-fold. Significantly changed genes were subjected to Gene ontology search using David Bioinformatics software (https://david.ncifcrf.gov).

Real-time quantitative reverse transcription–PCR (qRT-PCR)

Total RNAs were isolated from MTs and SGs of appropriate larval and pupal stages of the desired genotypes using TriReagent as per the manufacturer's (Sigma-Aldrich) instructions, followed by reverse transcription with Super-script Plus (Invitrogen, USA).The prepared cDNAs were subjected to real time PCR using forward and reverse primer pairs as listed below. Real time qPCR was performed using 5ml qPCR Master Mix (Syber Green, Thermo Scientific), 2 picomol/ml of each primer per reaction in 10 ml of final volume in ABI 7500 Real time PCR machine.

(i) reaper: Forward: 5'- CATACCCGATCAGGCGACTC-3'
Reverse: 5'- ACATGAAGTGTACTGGCGCA-3'
(ii) hid: Forward: 5'- AGCGCAGGAGACGTGTAATC -3'
Reverse: 5'- TTGTGTCCCGTCAACCTTCT -3'
(iii) drice: Forward: 5'- TGTCGGCCCACCCTTATCTA -3'
Reverse: 5'- TGGACGACCATGACACACAG -3'
(iv) dronc: Forward: 5'- ACGGGTGATTCACTGCCAAT -3'
Reverse: 5'- GACGCTCGTAGTTCGTCCAT -3'
(v) Atg8: Forward: 5'- AAGATCCGGCGCAAGTATCC -3'
Reverse: 5'- CGTCGGGACGCAGATTGATA -3'
(vi) E93: Forward: 5'- CGATTTGGCGCACAACAAGA -3'
Reverse: 5'- TGGCATCCGTTTCGGAATCA -3'
(vii) BR-C: Forward: 5'- CAATGGCAATGGCAACGGA -3'
Reverse: 5'- CTGGAGCGATTGGCATCGT -3'
(viii) EO: Forward: 5'- CCGATTCCGATGACTACTGG -3'
Reverse: 5'- CCGATTCCGATGACTACTGG -3'
(ix) Fkh: Forward: 5'- CCGGCGTAAATGTCCTGAGT -3'
Reverse: 5'- TGATGGTGGTGATGTGTGGG -3'

Acridine orange (AO) staining

Cell death was assessed by Acridine Orange (AO) vital stain (Jiang *et al.,* 1997). SGs and MTs were dissected at desired stages in PBS. Unfixed tissues were incubated in AO (1mg/ml) for 3 min. Tissues were then briefly rinsed in PBS and observed immediately in a fluorescence microscope (Nikon E800) using a B-2A filter.

Antibodies and immunocytochemistry

MTs from different stages of larvae and pupae were dissected in 1X PBS, fixed in 4% paraformaldehyde for 20 min at room temperature, rinsed in 0.1% PBST (1XPBS,0.1%TritonX-100), blocked in blocking solution (0.1% TritonX-100, 0.1%BSA, 10%FCS, 0.1% deoxycholate, 0.02% thiomersol) for 2h at room temperature. Tissues were incubated in primary antibody at 4 °C overnight. After three 0.1% PBST (20 min each) washings, tissues were blocked for 2h and incubated in the secondary antibody. Tissues were rinsed in 0.1% PBST and counterstained with DAPI (1mg/ml, Molecular Probe) for 15 min at room temperature. Washing was done again in 0.1%PBST and mounted in antifadant, DABCO (Sigma). Primary antibodies used were anti-LaminB (1:20, DSHB), anti-Caspase3 (1:100, Sigma). All preparations were analyzed under Zeiss LSM510MetaConfocal microscope and images were arranged and labeled using Adobe Photoshop7.

Acknowledgments

We thank Bloomington Drosophila *Stock Center, Dr. J.A.T. Dow, Dr. Eric Baehrecke and Dr. Ho Lam Tang for sharing their fly stocks. This work was supported by research grant from Department of Science and Technology and Department of Biotechnology Government of India, New Delhi. We also*

thank Department of Science and Technology, India, for providing National Facility for confocal microscope. Shainy Ojha was supported by a research fellowship from the Council of Scientific and Industrial Research, New Delhi.

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